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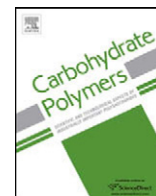
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# Starch modification with microbial alpha-glucanotransferase enzymes

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## ABSTRACT

Starch is an agricultural raw material used in many food and industrial products. It is present in granules that vary in shape in the form of amylose and amylopectin. Starch-degrading enzymes are used on a large scale in the production of sweeteners (high fructose corn syrup) and concentrated glucose syrups as substrate for the fermentative production of bioethanol and basic chemicals. Over the last two decades  $\alpha$ -glucanotransferases (EC 2.4.1.xx), such as branching enzyme (EC 2.4.1.18) and 4- $\alpha$ -glucanotransferase (EC 2.4.1.25), have received considerable attention. These enzymes do not hydrolyze the starch as amylases do. Instead,  $\alpha$ -glucanotransferases remodel parts of the amylose and amylopectin molecules by cleaving and reforming  $\alpha$ -1,4- and  $\alpha$ -1,6-glycosidic bond. Here we review the properties of  $\alpha$ -glucanotransferases and discuss the emerging use of these enzymes in the generation of novel starch derivatives.

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## 1. Enzymatic starch conversion

Starch is the major dietary carbohydrate for humans, as rice, corn, tapioca, wheat and potato form a major part of our diet. It is an abundant storage carbohydrate found in the leaves, seeds, roots and tubers of many plants (Zeeman, Kossmann, & Smith, 2010). Green plants use sunlight to transform carbon dioxide and water into sucrose which is then polymerized into starch and stored as semi-crystalline granules inside amyloplast organelles. Plants have two types of starch, one is found in leaves and the other in seeds, roots and tubers. Transitory leaf starch is made during the day to store excess energy. During the night most of the transitory leaf starch is consumed again while some of it is converted into sucrose that is transported to seeds, roots or tubers where it is converted back into starch. This type of starch is meant for long term energy storage that can be consumed by the young seedling during the first stage of germination. Over thousands of years man has cultivated starch containing crops for maximum starch yield. Commonly used starch-containing crops are corn, wheat, potato, rice, rye, and barley.

Normal starches are composed of about 20% of amylose, a virtually linear polymer of  $\alpha$ -1,4 linked glucose residues, and about 80% of amylopectin, a branched polymer of  $\alpha$ -1,4 linked glucose residues with about 5%  $\alpha$ 1,6-glycosidic bonds (Zeeman et al., 2010). There are also starches that contain almost exclusively amylopectin (the so-called waxy variants) and a few corn varieties that have elevated amounts of amylose (the Hylon varieties that have up to 50% amylose). The semi-crystalline starch granules are insoluble in cold water but swell and fall apart when heated. Subsequent cooling of the heated starch slurry leads to the formation of a firm, opaque gel due to hydrogen bonding between parallel oriented amylose and or long side chains of amylopectin. This process, termed retrogradation, is irreversible resulting in an insoluble gel.

Besides serving as food, starches are also industrially processed into a series of derivatives that are applied in various industries such as oil drilling fluids, adhesives, paper and cotton coatings, or gelling, emulsifying and viscosifying agents in food products. Starches are also converted into various syrups such as high fructose corn syrup on industrial scale (Buchholz & Seibel, 2008; Crabb & Shetty, 1999; van der Maarel, van der Veen, Uitdehaag, Leemhuis, & Dijkhuizen, 2002) to be used as filling agents and/or sweeteners in, e.g. soft drinks. Glucose syrups derived from mainly corn or wheat starch have become the basic raw material for the bioethanol industry (Lin & Tanaka, 2006). Glucose production starts with the liquefaction of 30–35% dry solid starch slurry by jet-cooking at temperatures above 100 °C and a first treatment with a heat stable  $\alpha$ -amylase (EC 3.2.1.1) for a few minutes at 90 °C. The next step is saccharification using a thermostable glucoamylase (EC 3.2.1.3) and pullulanase (EC 3.2.1.41) at 60 °C, yielding over 97% of glucose

**Abbreviations:** AGTases, alpha-glucanotransferases; 4 $\alpha$ GT, 4- $\alpha$ -glucanotransferase (amylomaltase); BE, branching enzyme; CGTase, cyclodextrin glucanotransferase; GI, Glycemic Index.

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**Table 1**Starch products made by  $\alpha$ -glucanotransferase enzymes.

Product	Applications	Enzyme
Cycloamylose	Protein folding	4 $\alpha$ GT
Cyclic cluster dextrin (CCD)	Sport drink	BE
Cyclodextrins	Cholesterol removal	CGTase
Thermoreversible starch gel (Etenia™)	Gelatin/fat replacer	4 $\alpha$ GT
Slowly digestible starch	Slow glucose release	BE
Resistant starch	Food fiber	4 $\alpha$ GT followed by debranching
Highly branched starch	E.g. paper coating	BE
Highly branched amylopectin cluster	Slow glucose release	BE followed by maltogenic amylase

in about 72 h. This glucose syrup can then be converted into a glucose/fructose mixture using immobilized glucose isomerase (EC 5.3.1.5). The enzymatic production of glucose and glucose/fructose syrups from starch is a bulk process consuming large amounts of enzyme (Crabb & Mitchinson, 1997; Crabb & Shetty, 1999). The common feature of these enzymes is the hydrolysis of the  $\alpha$ -1,4- or  $\alpha$ -1,6-glycosidic linkages leading to the degradation of the amylose and/or amylopectin. Over the last 10 years the use of  $\alpha$ -glucanotransferases (AGTases), i.e. non-hydrolyzing starch-active enzymes, has received considerable attention leading to a number of new commercial products (Table 1) (Biwer, Antranikian, & Heinzle, 2002; Dermaux, Peptitjean, & Wills, 2007; Kaper, van der Maarel, Euverink, & Dijkhuizen, 2004; Le et al., 2009; Norman, Pedersen, Stanley, Stanley, & Richmond, 2007; Richmond et al., 2008; Takaha & Smith, 1999; van der Maarel et al., 2005).

The widespread use of starch and starch derivatives, especially the easily digestible forms in all kinds of food products contributes to overweight/obesity, thereby being an important risk factor of several well-fare diseases (Brennan, 2005; Govindji, 2006; Swinburn, Caterson, Seidell, & James, 2004). Starch is a staple food present in many products we eat daily (e.g. bread, pasta, tortillas). Also starch hydrolysis products such as maltodextrins, glucose syrups and high fructose syrups are used in many food products (e.g. tomato ketchup, soft drinks, candy). Starch used in food and feed products is mostly processed by heating, mixing, or homogenization destroying the granules and thereby making the amylose and amylopectin easily accessible for digestive enzymes. After consumption, starch is converted along our gastrointestinal tract all the way to glucose, the actual energy source of our body. Degradation is initiated by salivary  $\alpha$ -amylase, an endo-acting enzyme that randomly hydrolyzes  $\alpha$ -1,4-glycosidic linkages in the amylose and amylopectin chains. The degradation continues in the small intestine where glucose is formed that is then taken up in the blood. The Glycemic Index (GI) expresses the rate at which glucose appears in the blood after the consumption of a starch containing foods. Food products with a high GI are white bread, cooked pasta and boiled potatoes, while green unripe bananas or kidney beans represent low GI foods (Atkinson, Foster-Powell, & Brand-Miller, 2008).

Starches can be categorized by the rate at which glucose is formed and appears in the blood. Rapidly degradable starches are converted in the small intestine within the first 20 min of digestion, whereas slowly digestible starches take more time to degrade. Resistant starch is mostly not degraded in the small intestine and enters the large intestine where it is degraded by gut bacteria *via* fermentation. Three classes of digestible starches are distinguished (Englyst, Kingman, & Cummings, 1992; Zhang & Hamaker, 2009):

- Rapidly digestible starch (RDS): starch that is completely converted within the first 20 min of the digestion test.

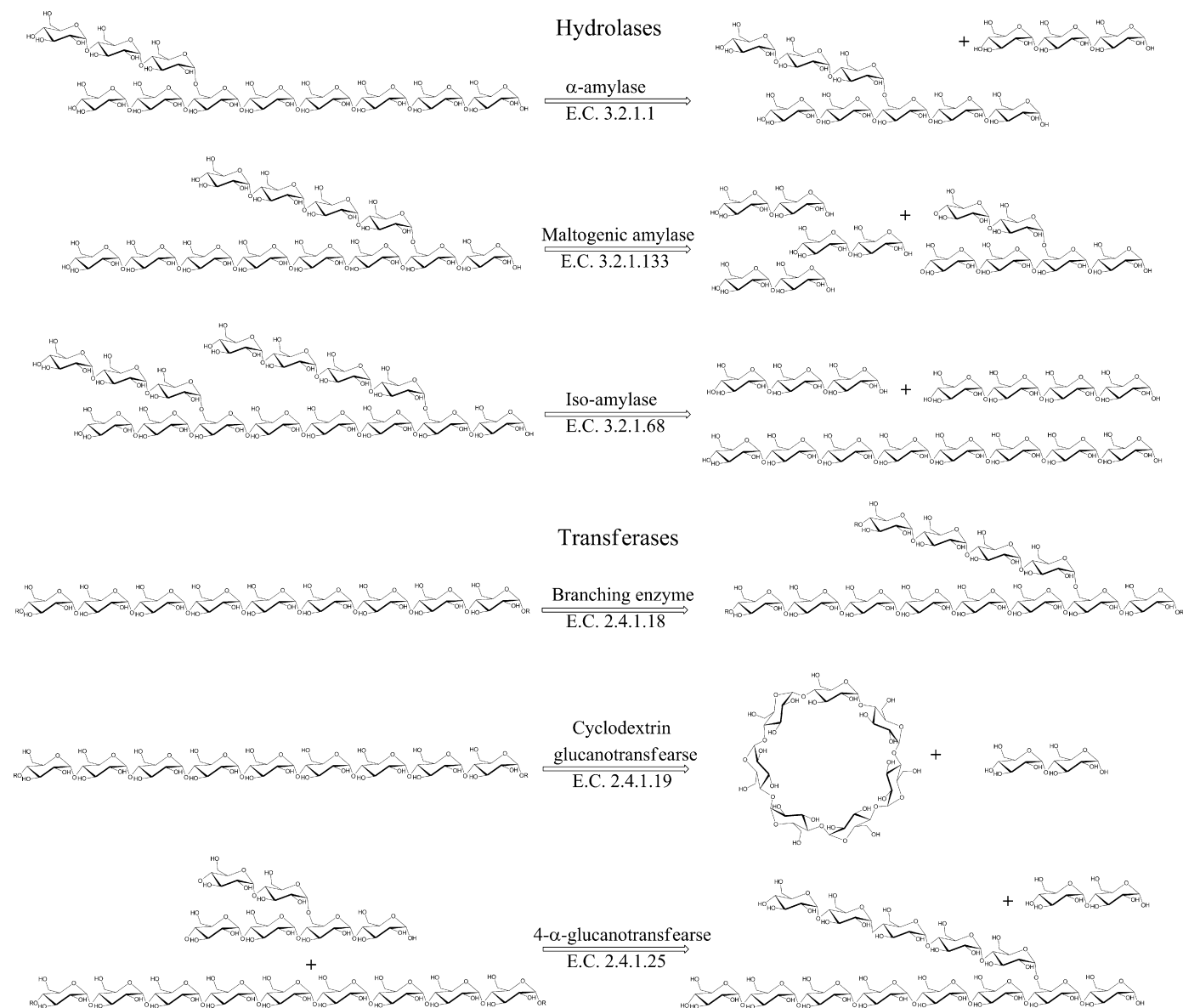
- Slowly digestible starch (SDS): starch that is degraded within a period of 120 min.
- Resistant starch (RS): typically defined as the sum of the unprocessed starch and its oligosaccharide degradation products that enter the colon.

Food scientist and technologists are continuously searching for starch processing methodologies that yield slower digestible starches. The view is emerging that AGTases enzymes can contribute to the generation of healthy starch based foods with respect to a controlled release of the glucose stored in the starch polymers.

## 2. The $\alpha$ -glucanotransferase enzymes

AGTases act on substrates with a number of consecutive  $\alpha$ -1,4-glycosidic linkages such as amylose, amylopectin, maltodextrins and glycogen. The crucial feature of AGTases is that they catalyze a disproportionation reaction, transferring the cleaved-off glucan to a glucan acceptor forming a new  $\alpha$ -glycosidic bond (Fig. 1). AGTases belong to the superfamily of glycoside hydrolases (GHs; for a comprehensive overview see [www.cazy.org](http://www.cazy.org)). GHs cleave glycosidic bonds and transfer the cleaved off fragment to an acceptor molecule, which is usually water resulting in a hydrolysis reaction, although some GHs transfer the cleaved off fragment to another sugar forming a new glycosidic bond in the so-called disproportionation (or transfer) reaction (Fig. 1). The AGTases discussed in this paper are found in three GH families, 13, 57 and 77 (Cantarel et al., 2009; Kuriki & Imanaka, 1999; Murakami, Kanai, Takata, Kuriki, & Imanaka, 2006; Stam, Danchin, Rancurel, Coutinho, & Henrissat, 2006; Zona, Chang-Pi-Hin, O'Donohue, & Janecek, 2004). Of these, the GH13 family is by far the largest and contains numerous hydrolases such as  $\alpha$ -amylase which plays a pivotal role in starch degradation, but also glycogen and starch branching enzymes and cyclodextrin glucanotransferases. The GH13, 57 and 77 enzymes are  $\alpha$ -retaining enzymes meaning that the newly formed glycosidic bond has the same anomeric configuration as the bond cleaved in the substrate. In these enzymes the chemistry is carried out by two acidic amino acids, one acting as nucleophile and one acting as acid/base (Vocadlo & Davies, 2008). Reactions start with the cleavage of an  $\alpha$ -1,4-glycosidic bond in the substrate resulting in a  $\beta$ -linked covalent glycosyl-enzyme intermediate. Following the departure of the glucan fragment from the acceptor subsites the non-reducing end of another glucan can enter the acceptor subsite +1 and attack the covalent intermediate with its 4-hydroxyl (4- $\alpha$ -glucanotransferases [4 $\alpha$ GTs] (EC 2.4.1.15) and cyclodextrin glucanotransferases [CGTases] (EC 2.4.1.19)) or 6-hydroxyl (starch and glycogen branching enzymes [BEs] (EC 2.4.1.18)), resulting in the formation of an  $\alpha$ -1,4- or  $\alpha$ -1,6-glycosidic bond, respectively.

Three types of AGTase enzymes are distinguished (i) 4- $\alpha$ -glucanotransferase (4 $\alpha$ GT; EC 2.4.1.25) sometimes indicated as amylomaltase, disproportionating or D-enzyme (Kaper et al., 2004), (ii) cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19) (Biwer et al., 2002; Leemhuis, Kelly, & Dijkhuizen, 2010) and (iii) branching enzyme (BE; EC 2.4.1.18), also known as Q-enzyme (Boyer & Preiss, 1977). Because AGTases use polymeric substrates, the hydroxyl acceptor can also be located downstream on the glycosyl-enzyme intermediate leading to an intra-molecular transglycosylation generating a cyclic product. For CGTases intra-molecular transglycosylation is the dominant reaction producing  $\alpha$ -,  $\beta$ - and  $\gamma$ -cyclodextrins with 6, 7 or 8  $\alpha$ -1,4 linked anhydroglucose residues (Fig. 1). CGTases can also transfer the glucan intermediate to a second glucan chain, i.e. inter molecular transglycosylation, forming a linear product. The later reaction is only dominant at very high concentrations of glucan acceptors with a free 4-hydroxyl group. In contrast to CGTases, 4 $\alpha$ GTs preferably catalyze inter molecular



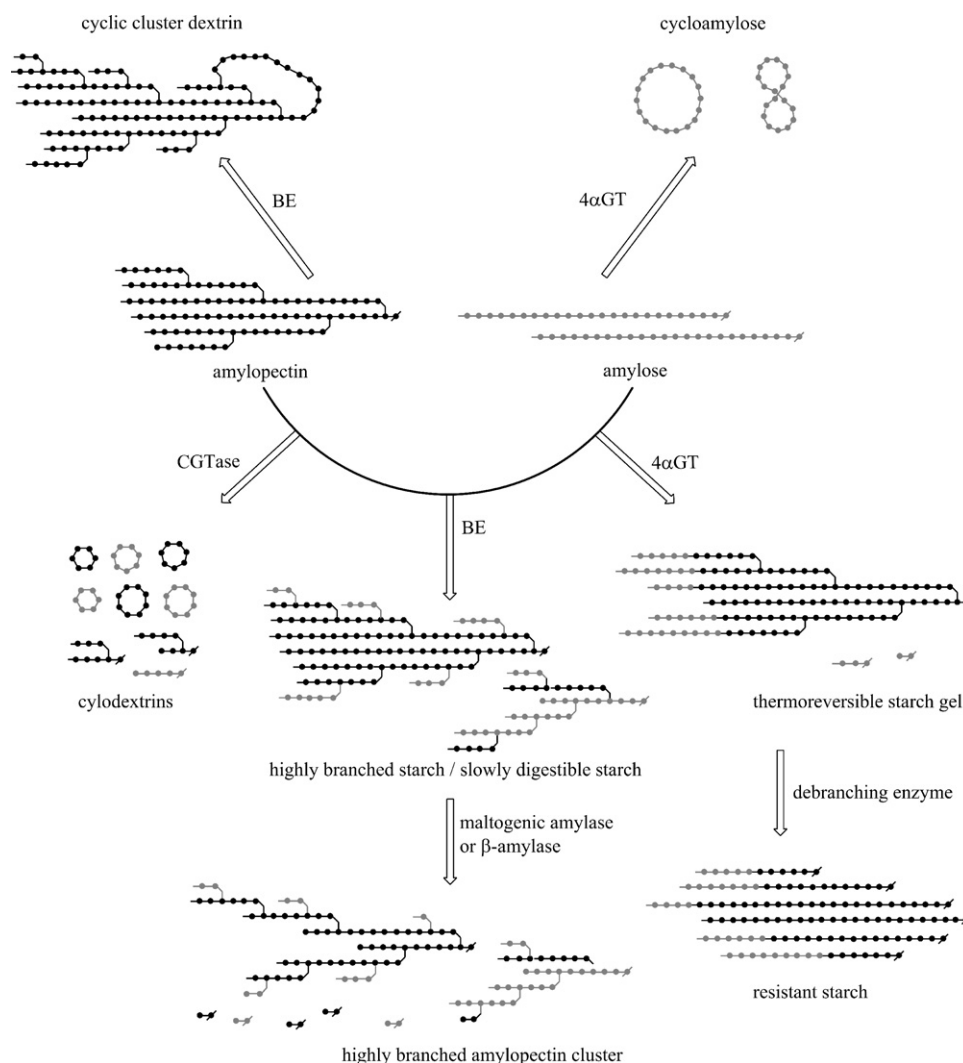
**Fig. 1.** Schematic representation of the reactions catalyzed hydrolases and transferases acting on starch.

transglycosylation, shuffling  $\alpha$ -1,4-glucan chains by cleaving and reforming  $\alpha$ -1,4-glycosidic bonds (Kaper et al., 2004) (Fig. 1). At low concentrations of acceptors bearing a 4-hydroxyl, 4 $\alpha$ GTs also catalyze intra-molecular transglycosylation forming cyclic molecules with 15 or more anhydroglucose residues (Terada, Fujii, Takaha, & Okada, 1999). BEs are quite different as they cleave an  $\alpha$ -1,4-glycosidic linkage and transfer an  $\alpha$ -glucan chain to a free 6-hydroxyl within a linear  $\alpha$ -1,4-glucan segment creating a branch (Fig. 1).

### 3. Glycogen branching enzyme products

In nature BEs create the  $\alpha$ -1,6-branches in the intracellular storage compounds glycogen and amylopectin (Zeeman et al., 2010). The fact that BEs break  $\alpha$ -1,4-glycosidic bonds and create  $\alpha$ -1,6-branches within linear  $\alpha$ -1,4 segments will ultimately result in products devoid of long  $\alpha$ -1,4 chains. BE are found in glycoside hydrolase families 13 and 57. The family GH57 BEs seem to act on amylose only (Palomo et al., 2011), while family GH13 BEs act on both amylopectin and amylose (Palomo, Kralj, van

der Maarel, & Dijkhuizen, 2009). BEs thus create products that form a viscous solution that cannot retrograde as the side-chains are too short to have a strong interaction. Retrogradation of the amylopectin fraction in bread limits the shelf-life of bread (Goesaert, Slade, Levine, & Delcour, 2009), and additives are commonly added during dough preparation in industrial produced breads. Although implicated (Spendler & Jorgensen, 1997), there is no convincing evidence reported that BEs are good anti-staling enzymes, nor have BE preparations been introduced on the market as anti-staling enzyme. Several patent applications describe the use of BEs for the production of highly branched  $\alpha$ -glucans (Fig. 2) that are slowly digestible, viscous starch derivatives for paper and textile industry (Bruinenberg, Hulst, Faber, & Voogd, 1996; Hendriksen, Pedersen, & Bisgaard-Frantzen, 1999), production of synthetic glycogen (Kajiura, Takata, Takaha, & Kuriki, 2006), food ingredients (Ohata, Yamamoto, Nakamura, Fujita, & Nakakuki, 2009; Sugimoto, Okada, Kitahata, Yoshikawa, & Sugimoto, 1982; Toda et al., 1985) and cyclic cluster dextrin (CCD). The CCD is made from amylopectin as the sole substrate by the action of glycogen BEs (Fig. 2) (Takata, Ohdan, Takaha, Kuriki, & Okada, 2003).



**Fig. 2.** The various products made by alpha-glucanotransferases from amylopectin and/or amylose. 4αGT, 4-α-glucanotransferase; BE, branching enzyme; CGTase, cyclodextrin glucanotransferase.

Only recently, a commercial glycogen BE with the trade name Branchzyme of Novozymes has become available. Branchzyme contains the thermostable glycogen BE of the thermophilic bacterium *Rhodothermus obamensis* (Shinohara et al., 2001). It was the first real glycogen BE reported to be active at temperatures of around 65 °C, a temperature at which the processing of gelatinized starch is feasible. Another thermostable glycogen BE used for industrial starch processing originates from the hyperthermophilic bacterium *Aquifex aeolicus* (Takata et al., 2003; van der Maarel, Vos, Sanders, & Dijkhuizen, 2003). This enzyme is used in the production of CCD, a sport drink ingredient made by Ezaki Glico of Japan (Takaha et al., 1997; Takata et al., 2003).

Regular starches are rapidly degraded by our digestive system, though they can be converted into slowly digestible starches by the actions of BEs. Maarel, Binnema, Semeijn, and Buwalda (2007) showed that the BE of the bacterium *Deinococcus radiodurans* (Palomo et al., 2009) converts amylopectin into a branched maltodextrin with a side chain distribution dominated by small side chains compared to other BEs. *In vitro* digestion analysis indicated that the product showed a slower glucose release when incubated with a pancreatic amylase preparation.

In the same framework the French company Roquette has developed a slow digestible starch by BE modification followed by β-amylase treatment (EC 3.2.1.2) (Fig. 2) (Dermaux et al., 2007). The

later enzyme is an exo-acting enzyme that cannot bypass branches and as such trims the side-chains by cutting of maltose units from the non-reducing ends. It was shown that the shorter side chains make the product less prone to α-amylase degradation and thus gives a slower glucose production in time. Instead of β-amylase maltogenic amylase (EC 3.2.1.133) can also be used to trim the side chains (Le et al., 2009).

#### 4. CGTase products

CGTases are extracellular enzymes that produce cyclodextrins from starch (Fig. 2), which are subsequently imported into the cell and there processed to supply the host with energy. CGTases are found in a wide array of bacteria and archaea living under various environmental conditions including high temperature (Bower et al., 2002; Kelly, Dijkhuizen, & Leemhuis, 2009b). CGTases were the first AGTases to be commercialized. A number of reviews on the production, properties and use of cyclodextrins have been published over the last years (Leemhuis et al., 2010; Martin Del Valle, 2004; Qi & Zimmermann, 2005). Cyclodextrins are mainly used because of their capacity to form inclusion complexes with various (hydrophobic) molecules. This encapsulation is advantageous to solubilize hydrophilic compounds in water and to lower the volatility of odor molecules. Cyclodextrins are also being used as



additives to mask undesirable tastes and to extract cholesterol from foods (Szente & Szejtli, 2004). CGTase have also been implicated in prevention of staling of bakery products, basically because the enzyme is structurally very similar to the commercial anti-staling enzyme Novamyl of Novozymes. Novamyl is a maltogenic amylase of a *Bacillus stearothermophilus* that cleaves of maltose units from the non-reducing end of the amylopectin side-chains, which slows down retrogradation. Shortening of a loop near the active changed Novamyl into a CGTase (Beier et al., 2000). CGTases, and mutant CGTases, have also been reported to improve the quality of baked goods (Kelly, Dijkhuizen, & Leemhuis, 2009a; Shim et al., 2007).

## 5. 4 $\alpha$ GT products

4 $\alpha$ GTs transfer fragments of amylose or parts of amylopectin side chains onto the non-reducing ends of amylopectin with the formation of new  $\alpha$ -1,4-glycosidic linkages (Fig. 2). In nature 4 $\alpha$ GTs participate in the intracellular metabolism of glycogen in bacteria and in the formation of amylopectin during starch biosynthesis in plants (Kaper et al., 2004). The 4 $\alpha$ GT of *Thermus thermophilus* is used to convert starch into a thermoreversible gelling derivative that is marketed under the trade name Etenia<sup>TM</sup> by AVEBE of The Netherlands (Euverink & Binnema, 1998). Gelatinized starch is incubated with a relatively low amount of enzyme and during relatively long incubation times. Etenia<sup>TM</sup> is free of amylose and has an amylopectin with short and relatively long side chains (van der Maarel et al., 2005). It is the presence of the long side chains, with a length of 35 glucose residues and longer, in combination with its relatively high molecular weight that makes Etenia<sup>TM</sup> to form a gel. However, in contrast to regular starch, this gel is thermoreversible; Etenia<sup>TM</sup> can undergo cycles of heating and cooling in which it dissolves upon heating and gels upon cooling. The gel strength and paste flow behavior of the 4 $\alpha$ GT-treated starches are related to the incubation conditions and enzyme to starch ratio (Hansen, Blennow, Pedersen, Norgaard, & Engelsen, 2008; Lee, Kim, Park, & Lee, 2008), whereas the melting temperature is independent of the reaction conditions and enzyme dosage. The thermoreversible gelling properties make Etenia<sup>TM</sup> similar to gelatin, which is used widely in food products as a gelling or viscosifying agent. However, its animal origin makes gelatin unacceptable for many customers. Besides being a good alternative to gelatin in, e.g. gum confectionary (Buwalda & Tomaso, 2009), Etenia<sup>TM</sup> can also be used to create a creamy texture in low fat products (Alting et al., 2009; Buwalda & Sein, 2008; Sein et al., 2009). After gelling, small particles are formed that have a size similar to that of fat droplets. When these particles come into contact with salivary amylase they fall apart. 4 $\alpha$ GT treated rice starch in the presence of 0.1% xanthan gum was also found to be a good fat replacer in mayonnaise (Mun et al., 2009).

Another product made by 4 $\alpha$ GTs is cycloamylose from amylose (Fig. 2) (Terada et al., 1999). Cycloamyloses are large cyclic compounds consisting of 16 or more  $\alpha$ -1,4 linked anhydroglucose residues. Because of their size, cycloamyloses form two or more helices in anti-parallel arrangement. Along the axis runs a hydrophobic channel that creates a hydrophobic interior in which hydrophobic molecules can be included such as certain drugs (Tomono et al., 2002). Another application of cycloamyloses is its use as artificial chaperones that help with the proper folding of heterologously produced proteins/enzymes (Machida et al., 2000). Ezaki Glico of Japan has developed the production of cycloamylose from amylose.

The sequential use of an 4 $\alpha$ GT and a debranching enzyme such as pullulanase leads to a resistant starch like product (Fig. 2) (Norman et al., 2007; Richmond et al., 2008). The debranched products consist of linear malto-oligosaccharides of such a degree of polymerization that they form crystallites. These crystallites are

inaccessible to salivary and pancreatic  $\alpha$ -amylases and thus end up in the large intestine. Tate & Lyle, Decatur USA, has developed this new type of resistant starch and markets them under the name of Promitor<sup>TM</sup> RS60 and RS75. Both resistant starches were shown to lower glycemic and insulin responses, compared to glucose and soluble fibers, in healthy volunteers (Kendall et al., 2008).

## 6. Conclusions

It is difficult to predict whether all of the proposed enzymatic starch processing technologies will be implemented by the food industry, but it is fair to say that the Etenia<sup>TM</sup> starch made by 4 $\alpha$ GT treatment is commercially available and is increasingly being used as food ingredient. In future novel enzyme activities that are able of slowing down the degradation of starch in the gastrointestinal tract may create new routes toward healthy starches. Such enzymes can thus replace chemical steps that introduce cross-linkages in starch.

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